JAI

Monoclonal Antibodies Derived from BALB/c Mice Immunized with Apoptotic Jurkat T cells Recognize Known Autoantigens

Timothy J. Gensler[1], Maria Hottelet[1], Chonghui Zhang[2], Stuart Schlossman[2], Paul Anderson[1] and Paul J. Utz[1,3]

[1] The Department of Medicine, Division of Rheumatology, Immunology and Allergy, Brigham & Women's Hospital, Boston, MA 02115, USA

[2] Department of Medicine, Division of Tumor Immunology, Dana Farber Cancer Institute, Boston, MA 02115, USA

[3] The Department of Medicine, Division of Immunology and Rheumatology, Stanford University School of Medicine, Stanford, CA 94305, USA

It has been postulated that post-translational modifications and relocalization of proteins during apoptosis may lead to presentation of these molecules to the immune system in such a way that normal mechanisms of tolerance are bypassed. In the present study, Jurkat cells were induced to undergo apoptosis by treatment with the chemotherapeutic agent Ara-C. BALB/c mice were then immunized with the apoptotic cells and hybridomas were generated. Using an indirect immunofluorescence assay, the monoclonal antibodies produced were screened by flow cytometry for those monoclonal antibodies demonstrating reactivity with permeabilized apoptotic Jurkat cells but not with non-permeabilized normal or apoptotic Jurkat cells. Of 281 monoclonal antibodies, 20 monoclonal antibodies with these properties were selected for further analysis. Using ^{32}P- or ^{35}S-metabolically labelled Jurkat cells, these selected monoclonal antibodies were screened for their ability to recognize auto-antigens by immunoprecipitation and Western blotting. Well characterized autoimmune sera were then used to confirm the identity of autoantigens by immunoblotting. We demonstrate that immunization of normal mice with apoptotic Jurkat cells results in the formation of antibodies targeting multiple autoantigens or autoantigen complexes, including Ku, rRNPs, snRNPs and vimentin. These findings are consistent with the hypothesis that apoptosis can contribute to the development of autoimmunity. © 2001 Academic Press

Introduction

Since the initial observations of several investigators that autoantigens relocalize to the cell surface in response to lethal stimuli such as ultraviolet (UV) irradiation and activation of death receptors such as Fas and the tumour necrosis factor (TNF) receptor, there has been great interest in identifying the role played by apoptosis in initiating autoimmunity [1–3]. It has been postulated that post translational modifications of autoantigens during apoptosis may somehow be involved in the genesis of autoantibodies in several diseases, including SLE, scleroderma and dermatomyositis [review in 4, 5]. Many autoantigens are cleaved *in vivo* and *in vitro* by a family of proteases (called caspases because they cleave substrates immediately following an aspartic acid residue within a particular local context) [6, 7]. Another protease, the cytotoxic-granule protein granzyme B, has recently been shown by Casciola-Rosen and colleagues to cleave many autoantigens at sites not usually recognized by caspases [8, 9]. Other modifications of autoantigens such as RNA cleavage [10–12]. DNA cleavage [13] and methylation [14], and protein transglutamination [15], citrullination [16], phosphorylation [17, 18], dephosphorylation [19], and ubiquitination [20] also occur during cell death.

If apoptotic cells are an important reservoir of autoantigens from which the loss of tolerance to self-antigens originates, then exposure to apoptotic cells might be expected to result in autoantibody production. Importantly, when normal mice were immunized with syngeneic apoptotic cells, their sera developed low levels of autoantibodies, whereas the sera from control mice immunized with non-apoptotic cells generally did not contain autoantibodies [21]. These investigators looked only at serum from these mice; to date there have been no studies in which monoclonal antibodies rather than sera derived from mice immunized with apoptotic cells, have been analysed. The use of monoclonal antibodies rather

Correspondence to: P. J. Utz, M.D., Stanford University School of Medicine, Division of Immunology and Rheumatology, CCSR Building, Room 2215-A, 300 Pasteur Drive, Stanford, CA 94305, USA. Fax: (650) 723-3509. E-mail: pjutz@stanford.edu

than sera permits the examination of individual components of an immune response without one component confounding another.

In an effort to strengthen the potential relationship between apoptosis and the development of antibodies targeting autoantigens, we immunized BALB/c mice with apoptotic Jurkat cells and generated monoclonal antibodies. Using flow cytometry, we selected 20 monoclonal antibodies which demonstrated reactivity with permeabilized apoptotic Jurkat cells but not with non-permeabilized normal or apoptotic Jurkat cells. Characterization of the 20 monoclonal antibodies is described, and the implications of the results as they relate to programmed cell death and the loss of tolerance to self-antigens is discussed.

Materials and Methods

Generation of monoclonal antibodies

Monoclonal antibodies were generated by immunizing BALB/c mice with apoptotic human Jurkat T cells as previously described [22]. Briefly, three female BALB/c mice were immunized four times, either subcutaneously (sc) or intraperitoneally (ip) at 3- to 4-week intervals with Jurkat cells that had been treated with 1-β-d-arabinofuranosylcytosine (Ara-C) (Sigma Chemical Co., St Louis, MO, USA) to induce apoptosis. Three days before cell fusion, the mice were boosted by ip injection with the apoptotic Jurkat cells. Splenocytes from the immunized mouse were fused with myeloma cells using polyethylene glycol by the method previously described [23]. Approximately 850 hybridomas were generated in total from the three separate fusions.

Monoclonal antibody screening using flow cytometry

Jurkat T cells were permeabilized by the method previously described [24, 25] with minor modifications [22]. Briefly, cells were fixed in 1% formalin solution in PBS for 20 min on ice and washed twice with PBS before the cells were permeabilized by incubation on ice with digitonin (Aldrich, Milwaukee, WI, USA). A digitonin stock solution of 10 mg/ml was prepared in DMSO, and a final digitonin concentration of 10 µg/ml diluted from the stock solution with PBS was used for cell permeabilization. The permeabilization of cells by digitonin was confirmed by the uptake of trypan blue. After permeabilization, the cells were washed and resuspended in PBS for immunofluorescence staining. FACS analysis for each antibody was performed on Jurkat cells that were (i) non-apoptotic, permeabilized with digitonin; (ii) non-apoptotic, non-permeabilized with digitonin; (iii) apoptotic, permeabilized with digitonin; and (iv) apoptotic, non-permeabilized with digitonin. Of the original 850 antibodies, 281 recognized Jurkat cells that had been treated and processed as above (i and iii

but not ii or iv). Twenty of these 281 antibodies were randomly selected for this study.

Cells subjected to digitonin permeabilization or mock permeabilization were labelled with each monoclonal antibody for flow cytometry by an indirect immunofluorescence assay. After incubation on ice for 40 min with 100 µl of monoclonal antibody supernatant or diluted ascites, cells (~1×10^6 cells/sample) were washed three times with PBS containing 0.1% bovine serum albumen (BSA, Intergen Co., Purschase, NY, USA) and 0.01% NaN$_3$. After a further incubation for 40 min with affinity-purified goat anti-mouse IgG- or IgM-FITC conjugate (1:500) (Jackson Immuno-Research, West Grove, PA, USA), the cells were washed three times in the buffer above and fixed in 1% formalin solution in PBS for flow cytometric analysis.

Cell culture

Jurkat cells were grown in 5% CO$_2$ at 37°C using RPMI 1640 (Bio Whittaker, Walkersville, MD, USA) supplemented with 9% heat-inactivated fetal calf serum (HI-FCS) (Tissue Culture Biologicals, Tulare, CA, USA) and penicillin and streptomycin (Mediatech, Inc., Herndon, VA, USA). Jurkat cells were grown and harvested at mid-log phase.

Metabolic labelling

Jurkat cells were incubated at a density of 2×10^6 cells/ml in labelling medium containing the following: 90% RPMI 1640 lacking methionine and cysteine (Gibco, Grand Island, NY, USA) and 10% HI-FCS that had been dialysed to equilibrium against 10 mM HEPES buffer (Sigma Chemical Co.). ^{35}S-labelled methionine and cysteine label (Dupont, New England Nuclear, NEN, Boston, MA, USA) was added at a concentration of 0.1 mCi/ml. Cells were incubated at 37°C for 2 h to allow the cells to reach steady-state before each treatment, unless otherwise indicated. Cells were harvested at the end of the time course to ensure equal labelling for each treatment.

Cell lysis

Jurkat cells were solubilized in Nonidet P-40 (NP40) (Sigma Chemical Co.) lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris pH 7.8, 1 mM EDTA). NP40 lysis buffer was supplemented immediately before use with a 100× protease inhibitor cocktail containing chymostatin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, benzamidine and aprotinin, prepared as described [26]. All chemicals were purchased from Sigma. After addition of lysis buffer, cells were incubated on ice for 30–60 min, centrifuged in a refrigerated microfuge (5402; Eppendorf, Hamburg, Germany) at 14,000 rpm for 15 min, and the supernatant used immediately for each experiment.

Cellular activation

Radiolabelled Jurkat cells were treated with anti-Fas antibody 7C11 (IgM; kindly provided by Michael Robertson, Indiana University, Bloomington, IN, USA) prepared from hybridoma supernatant (1:500 v:v). Cells were incubated at 37°C for the indicated times prior to harvesting.

Immunoprecipitation and Western blot analysis

Lysates were precleared once with 20 µl of a 50% solution of Protein A Sepharose (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline (PBS) and 5 µg rabbit anti-mouse (RAM) IgG (Jackson Immuno-Research) for 1 h, followed by two preclears with Protein A Sepharose overnight. Mouse monoclonal antibodies (2 µl) and 5 µg RAM IgG or IgM, or 2 µl patient serum alone were used in precipitation experiments. For monoclonal antibodies Z12, Z13, Z14 and Z15, 500 µl of serum-free culture supernatant was also used in separate precipitation experiments. Human polyclonal antibodies were obtained from the following sources and stored at −70°C until used: P. Fraser, Brigham and Women's Hospital, Boston, Massachusetts: healthy human control; Arthritis Foundation/CDC Reference Sera, Atlanta, Georgia: anti-Sm, anti-U1 RNP; E. Tan and C. Casiano, The Scripps Research Institute, La Jolla, California: anti-ribosomal P (serum KH); Immunovision Inc., Springdale, Arizona: anti-ribosomal P. Mouse monoclonal antibodies were obtained from the following sources and stored at −70°C until used: D. Weaver, Center for Blood Research, Boston, Massachusetts: anti-DNA-PK and anti-Ku70; W. J. van Venrooij, Katholieke Universiteit Nijmegen, The Netherlands: anti-U1A/U2B" (9A9) [27]; Immunoprecipitations from cell lysates were performed in NP40 lysis buffer in a total volume of 500 µl and rotation in a 4°C cold room for 2–24 h. Precipitates were harvested by centrifuging for 25 s at 14,000 rpm in a refrigerated Eppendorf microfuge, washing three times with NP40 lysis buffer, resuspending in SDS loading buffer with 9% 2-mercaptoethanol, boiling for 5 min, and separating by SDS PAGE as described [28]. Proteins were transferred to nitrocellulose (Schleicher and Scheull, Keene, NH, USA) and either exposed for autoradiography or subjected to Western blot analysis as indicated. Antibodies were used at the following concentrations: mouse monoclonal antibody Z7 (anti-Ku 70), 1:1,000; anti-ribosomal P (KH serum), 1:2,000; anti-Sm (CDC/AF reference serum 5), 1:2,000; anti-vimentin antibodies (Sigma Chemical Co.), 1:500. Nitrocellulose blots were blocked with 5% Blotto (Biorad, Inc., Hercules, CA, USA) in PBS overnight at 4°C. Bands were visualized using species-specific antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL, USA) at a dilution of 1:7,500 in 5% Blotto in PBS, and developed using ECL chemiluminescence performed according to the manufacturer's instructions (Amersham), or using species-specific antibody conjugated to alkaline phosphatase and developing using colorimetric substrate as indicated.

Cloning of Ku 70

Monoclonal Z3 was used to screen a Human T cell cDNA library (Stratagene, Inc.). Of approximately 2 million plaques that were screened, four clones were positive. Two of the four clones were sequenced and used to screen GenBank™ sequences using the FASTA program [29] at the GENSTREAM network server IGH Montpellier, France. Alignment of open reading frames corresponding to the retrieved DNA sequences was performed using LALIGN.

Results

Monoclonal antibodies derived from mice immunized with apoptotic Jurkat cells precipitate proteins from lysates prepared from ^{35}S-labelled Jurkat T cells

Monoclonal antibodies were produced by immunizing BALB/c mice with apoptotic Jurkat T cells as previously described (see Methods) [22]. Subsequently, the monoclonal antibodies were screened by flow cytometry using an indirect fluorescence assay. Those monoclonal antibodies which demonstrated reactivity with permeabilized apoptotic Jurkat cells but not apoptotic or untreated non-permeabilized Jurkat cells were selected for further analysis. Of note, these selected monoclonal antibodies also demonstrated reactivity by flow cytometry with digitonin-permeabilized normal Jurkat cells (although less than with digitonin-permeabilized apoptotic Jurkat cells, Figure 1), suggesting that the antibodies recognized intracellular antigens. A summary of the antibodies and their properties is presented in Table 1, and FACS analyses of several representative antibodies (Z2 and Z8) is shown in Figure 1.

Jurkat cells petabolically labelled with ^{35}S methionine and cysteine were cultured over a 3-h period, solubilized in NP40 lysis buffer, and immunoprecipitated using ascites containing each monoclonal antibody, except for monoclonal antibodies Z12, Z13, Z14 and Z15 (Figure 2, lanes 12, 13, 14 and 15) where serum-free culture supernatants were used instead. Immunoprecipitates were separated on a 12% SDS polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiography. As shown in Figure 2, nearly half of the monoclonal antibodies precipitate labelled proteins in one of several unique patterns. For example, monoclonal antibodies Z3 and Z7 (lanes 3 and 7) immunoprecipitate a doublet of 70- and 80-kDa (p70, p80, left arrows), while monoclonal antibodies Z13 and Z15 (lanes 13 and 15) immunoprecipitate a similar pattern of proteins in a broad molecular weight range from approximately 5- to 90-kDa. Additionally, an approximately 58-kDa protein (p58,
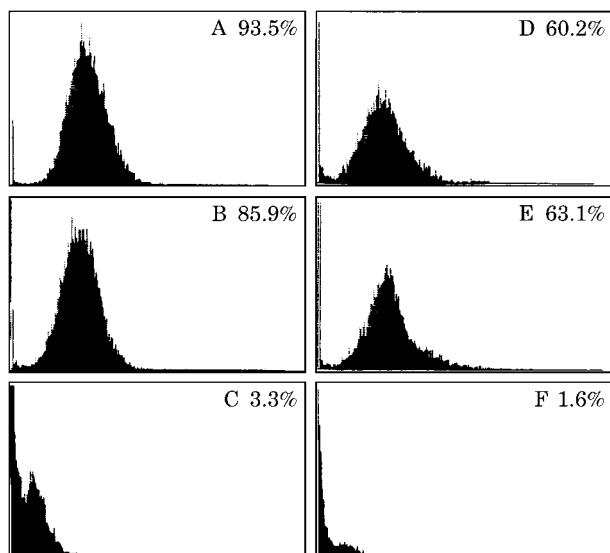
Figure 1. Flow cytometric profiles of monoclonal antibody staining of digitonin-permeabilized normal and apoptotic cells. Jurkat cells were induced to undergo apoptosis with Ara-C. Both apoptotic (A–C) and normal untreated (D–F) Jurkat cells were fixed in 1% formalin solution in PBS for 20 min and permeabilized by incubation on ice for 5 min with digitonin. Permeabilized Jurkat cells were stained with mAb Z2 (A and D), mAb Z8 (B and E), or a negative control antibody (C and F), followed by staining with affinity-purified goat anti-mouse IgG-FITC conjugate prior to flow cytometric analysis. The percentage of positive cells is indicated in each profile. Antibodies correspond to those listed in Table 1.

left arrows) can be seen in five immunoprecipitations (lanes 2, 4, 6, 12 and 14). These results suggest that immunization of mice with apoptotic Jurkat cells results in the production of antibodies capable of precipitating several protein complexes or particles in their native state.

Monoclonal antibodies immunoblot Ku 70

We next sought to determine if any of the monoclonal antibodies recognize denatured antigens by subjecting them to Western blot analysis using Jurkat cell lysates as a substrate. Jurkat cells were solubilized in Nonidet P-40 lysis buffer, separated on a 12% SDS-polyacrylamide gel, and transferred to nitrocellulose. Western blotting was carried out using ascites containing each monoclonal antibody separately. As shown in Figure 3, two monoclonal antibodies immunoblotted proteins, both of which were approximately 70 kDa (lanes 3 and 7). None of the other 18 monoclonal antibodies recognized proteins in this analysis despite the ability of nearly half of the antibodies to precipitate proteins (Figure 2). All 20 monoclonals were also screened for their capacity to immunoblot proteins present in lysates prepared from apoptotic Jurkat cells, and similar results were obtained (data not shown).

In order to determine the identity of the 70-kDa protein recognized by these two monoclonal antibodies, a human T cell cDNA library was screened

using ascites containing monoclonal antibody Z3. Of two million clones screened, four were positive. Surprisingly, sequence analysis of two of the clones revealed that they both encoded the human Ku 70 protein. Immunoprecipitation of unlabelled Jurkat cell lysates using monoclonal antibody Z7 followed by separation on a 12% SDS-polyacrylamide gel, transfer to nitrocellulose and immunoblotting using monoclonal antibody Z3 demonstrated a 70-kDa protein (Figure 4, lane 5). Reciprocal experiments using monoclonal antibody Z3 for the immunoprecipitation and monoclonal antibody Z7 for the immunoblotting produced identical results, suggesting that monoclonal antibody Z7 is also an anti-Ku 70 antibody. These findings are congruent with the ^{35}S methionine and cysteine-labelled immunoprecipitates by these two monoclonals (Figure 2, lanes 3 and 7) which demonstrate two very prominent proteins migrating at 70 and 80 kDa. In addition, a high molecular weight protein present in the immunoprecipitates is recognized by antibodies directed against DNA-dependent protein kinase (anti-DNA-PK, data not shown), suggesting that Z3 and Z7 co-immunoprecipitate a complex consisting of Ku 70, Ku 80, and DNA-PK. Interestingly, in over five separate experiments, a number of other antibodies (e.g., Z4, Z6, Z12, and Z13, Figure 4, lanes 3, 4, 7 and 8) were also capable of immunoprecipitating weak bands that were recognized by an anti-Ku monoclonal antibody. None of the other antibodies listed in Table 1 exhibited any ability to precipitate Ku 70 (data not shown). In most cases these additional antibodies immunoprecipitate very weak or absent bands from radiolabelled Jurkat cell lysates (e.g., Figure 2, lanes 4, 6, 12, and 13). This is likely the result of variable metabolic labelling between experiments, as the Western blot analysis shown in Figure 4 was performed in a separate experiment from that shown in Figure 2. The finding that a known autoantigen complex was recognized by a subset of these monoclonal antibodies generated against apoptotic Jurkat cells led us to carefully examine the possibility that other autoantigens are also targeted by this panel of monoclonal antibodies.

Monoclonal antibodies precipitate ribosomal protein autoantigens from Jurkat T cell lysates

Examination of the pattern of proteins immuno-precipitated by two of the monoclonal antibodies, Z13 and Z15, using either [35]S- (Figure 2, lanes 13 and 15) or [32]P- (data not shown) labelled Jurkat cell lysates, resembled the published pattern of ribosomes when separated by SDS–PAGE [30]. Therefore, these two monoclonal antibodies were further evaluated to determine if they precipitated ribosomal P auto-antigens. Jurkat cells were solubilized in Nonidet P-40 lysis buffer and immunoprecipitated using human anti-ribosomal P antibody (Immunovision, Inc., Figure 5, lane 1); healthy control human serum (Figure 5, lane 4); and ascites containing the two selected monoclonal antibodies Z13 and Z15 (Figure 5, lanes 2 and 3). The immunoprecipitates were

Table 1. *Characterization of individual monoclonal antibodies*

Monoclonal	Isotype	Ribo P	snRNP	Vimentin	Immunoblot
Z1	IgA	N.D.	N.D.	−	−
Z2	IgG	N.D.	−	?	−
Z3	IgG1	−	−	−	Ku70
Z4	IgM	N.D.	−	++	−
Z5	N.D.	N.D.	N.D.	−	−
Z6	IgG1	N.D.	−	++	−
Z7	IgG1	N.D.	−	−	Ku70
Z8	IgG1	N.D.	N.D.	N.D.	−
Z9	IgG1	N.D.	N.D.	N.D.	−
Z10	N.D.	N.D.	−	N.D.	−
Z11	IgG1	N.D.	−	−	−
Z12	IgM	N.D.	?	+	−
Z13	IgG1	+	?	−	−
Z14	IgM	N.D.	?	++	−
Z15	IgM	+	?	−	−
Z16	IgM	N.D.	N.D.	−	−
Z17	IgM	N.D.	N.D.	N.D.	−
Z18	IgM	N.D.	N.D.	N.D.	−
Z19	IgG1	N.D.	N.D.	N.D.	−
Z20	IgM	N.D.	N.D.	N.D.	−

Individual monoclonal antibodies are identified by the Z-number in each row. The specific antibody isotype is indicated for each monoclonal antibody. The following indicate that the noted monoclonal antibody immunoprecipitates the specified protein complex as determined by Immunoprecipitation/Western blot analysis: Ribo P, ribosomal P autoantigens rRNP1, rRNP2, and rRNP3; snRNP, U1-small nuclear ribonucleoprotein; vimentin. Immunoblot indicates the specific immunoreactivity of the indicated monoclonal antibody against Jurkat cell lysates. Test results are labeled as positive (+); strongly positive (++); negative (−); inconclusive (?); not done N.D.
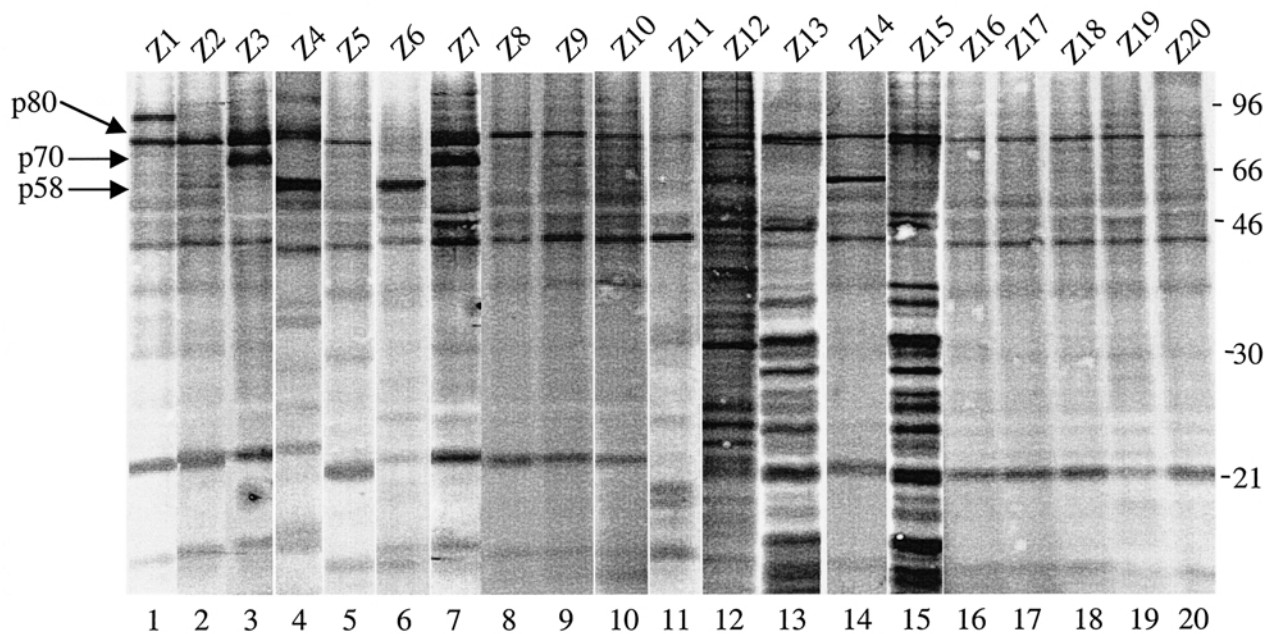

Figure 2. Analysis of proteins immunoprecipitated from radiolabelled Jurkat cell lysates using monoclonal antibodies. Jurkat cells were labelled with ^{35}S methionine and cysteine and subsequently solubilized in Nonidet P-40 lysis buffer. Proteins were precipitated using the indicated monoclonal antibodies, separated on 12% SDS–PAGE gels, transferred to nitrocellulose and exposed for autoradiography. Bands corresponding to proteins p58, p70 and p80 are indicated on the left side of the panel. The relative migration of molecular mass markers in kilodaltons is on the right side of the panel. Lanes are numbered at the bottom of the figure. Antibodies correspond to those listed in Table 1.

separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Cell lysate was also separated on the gel (lane 5). The immobilized proteins were immunoblotted using human anti-ribosomal P antibody (KH serum, a gift of C. A. Casiano and E. M. Tan). As shown in Figure 5, lanes 2 and 3, the two
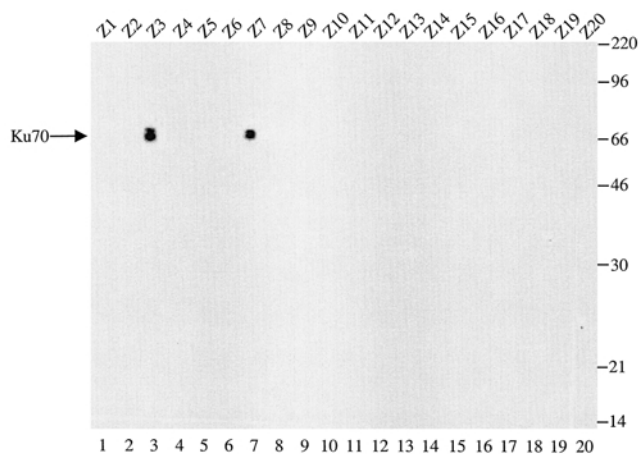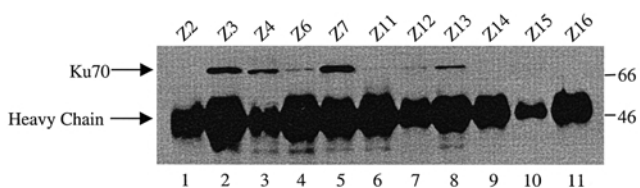
Figure 3. Western blot analysis of monoclonal antibodies. Jurkat cells were solubilized in Nonidet P-40 lysis buffer, separated on a 12% SDS–PAGE gel and transferred to nitrocellulose. Each monoclonal antibody was assessed for its ability to immunoblot immobilized proteins. A band corresponding to Ku 70 is labelled on the left side of the panel. The relative migration of molecular mass markers in kDa is indicated on the right side of the panel. Lanes are numbered at the bottom of the figure. Antibodies correspond to those listed in Table 1.

Figure 4. Monoclonal antibodies immunoprecipitate Ku 70 from Jurkat cell lysates. Jurkat cells were solubilized in Nonidet P-40 lysis buffer and immunoprecipitated with the indicated monoclonal antibody. After separation on a 12% SDS-polyacrylamide gel and transfer to nitrocellulose, the filter was immunoblotted using anti-Ku monoclonal antibody (Z3). The band corresponding to Ku 70 is indicated on the left side of the panel. The relative migration of molecular mass markers in kilodaltons is indicated on the right side of the panel. Lanes are numbered at the bottom of the panel. Antibodies correspond to those listed in Table 1.

monoclonal antibodies precipitate all three ribosomal P autoantigens (rRNP 1, rRNP2 and rRNP3, left arrows). Healthy control serum fails to immunoprecipitate these autoantigens (lane 4) while the positive control anti-ribosomal P serum immunoprecipitates all three rRNP autoantigens, as expected (lane 1). Thus, in addition to the Ku/DNA-PK particle, ribosomes are also recognized by monoclonal antibodies prepared from mice immunized with apoptotic Jurkat cells.

Monoclonal antibodies precipitate U-small nuclear ribonucleoprotein (U-snRNP) autoantigens from Jurkat T cell lysates

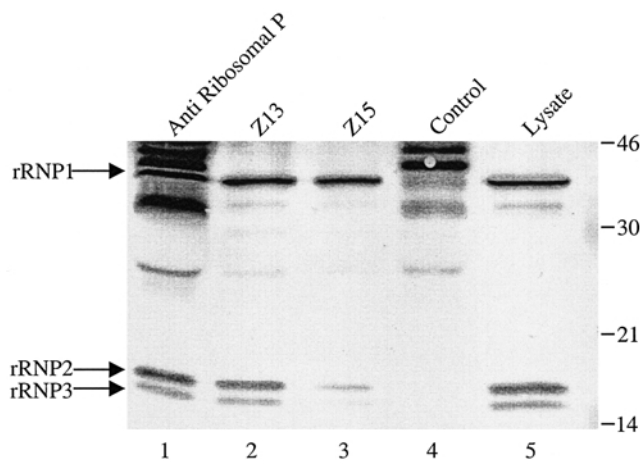Evidence that a second autoantigenic particle was recognized by two of the monoclonal antibodies

Figure 5. Monoclonal antibodies immunoprecipitate ribosomal protein autoantigens from Jurkat cell lysates. Jurkat cells were solubilized in Nonidet P-40 lysis buffer and immunoprecipitated with the indicated monoclonal antibody or human sera. After separation on a 12% SDS-polyacrylamide gel and transfer to nitrocellulose, the filter was immunoblotted using anti-ribosomal P (KH serum). Bands corresponding to ribosomal P autoantigens rRNP1, rRNP2, and rRNP3 are indicated on the left side of the panel. The relative migration of molecular mass markers in kilodaltons is indicated on the right side of the panel. Lanes are numbered at the bottom of the panel. Antibodies correspond to those listed in Table 1.

obtained from mice immunized using apoptotic cells directed us to search for other immunoprecipitated autoantigens. The appearance of the immunoprecipitation pattern produced by four of the monoclonal antibodies using cell lysates derived from ^{32}P-labelled apoptotic Jurkat cells (data not shown) was similar to that recently reported for phosphorylated serine/arginine (SR) splicing factors, which are known to associate with the U1-snRNP and U3-snoRNP autoantigen complexes during apoptosis [17, 18, 31]. Therefore, these four monoclonals were tested for their ability to immunoprecipitate U-snRNPs from Jurkat cell lysates. Jurkat cells were solubilized in Nonidet P-40 lysis buffer and immunoprecipitated using human anti-Sm (CDC/AF reference serum 5), (Figure 6, lane 7); monoclonal antibody U1A/U2B″ (9A9) [27], (Figure 6, lane 6); and ascites containing five selected monoclonal antibodies (Figure 6, lanes 1–5). Immunoprecipitates were separated on a 12% polyacrylamide gel, transferred to nitrocellulose and immunoblotted using anti-Sm (CDC/AF reference serum 5). Anti-Sm has been shown previously to immunoblot multiple proteins in the Smith complex including the protein doublet B/B′ [32]. As shown in Figure 6, the two bands corresponding to B and B′ (right arrows) are clearly present in immunoprecipitates prepared using monoclonal antibodies Z12, Z13, Z14, and Z15 (lanes 2–5), as well as monoclonal anti-U1A/U2B″ (lane 6) and anti-Sm (lane 7). The relative signal intensity of the B and B′ bands is less in the monoclonal antibody immunoprecipitates (lanes 2–5) compared to positive control anti-U1A/U2B″ and anti-Sm (lanes 6 and 7). These two bands are not present using Z8 (lane 1).
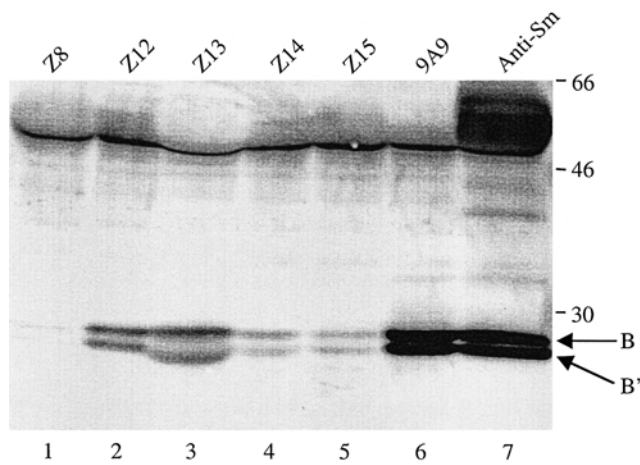
Figure 6. Monoclonal antibodies immunoprecipitate snRNP antigens from Jurkat cell lysates. Jurkat cells were solubilized in Nonidet P-40 lysis buffer and immunoprecipitated with individual monoclonal antibodies or human serum. Immunoprecipitates were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. The filter was immunoblotted with anti-Sm (CDC/AF reference serum 5). Bands corresponding to U-snRNP proteins B and B′ are indicated at the right side of the panel. The relative migration of molecular mass markers in kilodaltons is also indicated on the right side of the panel. Lanes are numbered at the bottom of the panel. Antibodies correspond to those listed in Table 1.

The lower signal intensity of B and B′ in the monoclonal antibody immunoprecipitates compared with the two positive control antibodies raises the possibility that the monoclonal antibodies have a low affinity for snRNPs or that the snRNPs are being coprecipitated, perhaps related to their interaction with RNA. In fact, incubation of ^{35}S-labelled Jurkat cell lysates with RNase prior to immunoprecipitation using the monoclonal antibodies did appear to significantly reduce the amount of protein precipitated in the case of monoclonal antibodies Z13 and Z15 but not with Z12 and Z14 (data not shown). This result suggests that the epitope recognized by some of these monoclonal antibodies requires RNA to achieve the necessary conformation for antibody binding, or that some of the precipitated proteins including snRNPs are coprecipitated by virtue of tethering to the same RNA molecule. The possibility that some of these antibodies directly recognize RNA has not been formally addressed in these studies. Finally, these monoclonal antibodies may recognize only a subset of U-snRNPs, a hypothesis that is supported by the ability of these antibodies to coprecipitate phosphorylated SR proteins from lysates prepared from untreated cells (data not shown), which normally are coprecipitated efficiently only when extracts are prepared from apoptotic cells [17, 18].

We used the standard technique of ip injection of pristane into BALB/c mice to obtain monoclonal antibody-enriched ascitic fluid. Pristane injection has been shown in several interesting reports to induce a number of unique autoantibodies including anti-Sm, anti-RNP, anti-ribosome, anti-Su, and anti-dsDNA. It

has been shown that this can result in the production of high-titre, specific anti-U-snRNP antibodies approximately two to four months following pristane priming, and in the absence of coinjection of hybridoma cells [33]. Since the antibodies used in our initial screen were prepared in mice that had been primed with pristane prior to instillation of individual hybridomas, and to control for the possibility that a pristane-induced artifact was responsible for the generation of antibodies directed against U-snRNPs, we performed comparable experiments using serum-free culture supernatants of Z12, Z13, Z14 and Z15. Identical results were obtained (data not shown). Taken together, these results eliminate the possibility of a 'pristane artifact', and suggest that a third auto-antigen complex, the U-snRNP, is precipitated by four of the monoclonal antibodies derived from mice immunized with apoptotic Jurkat cells.

Monoclonal antibodies immunoprecipitate vimentin

We noted that five monoclonal antibodies immunoprecipitated a 58-kDa protein from ^{35}S-labelled Jurkat cell lysates (Figure 2; lanes 2, 4, 6, 12 and 14). We were intrigued that this 58-kDa protein was precipitated by one-quarter of the monoclonal antibodies tested. Since these monoclonal antibodies were produced by immunizing mice using apoptotic Jurkat cells, we wondered whether an important modification of the 58-kDa protein during apoptosis was occurring and perhaps enhancing the protein's immunogenicity. Therefore, we investigated the effect of Fas-induced apoptosis on this protein. Jurkat cells metabolically labelled with ^{35}S methionine and cysteine were cultured over a 4-h period in the absence or presence of a monoclonal antibody reactive with Fas (anti-7C11), solubilized in Nonidet P-40 lysis buffer, and immunoprecipitated using these selected monoclonal antibodies. Immunoprecipitates were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose and subjected to autoradiography. DNA fragmentation in cells treated with anti-7C11 was confirmed in parallel experiments (data not shown). As shown in Figure 7, a 58-kDa band is observed (lanes 1, 3, 5, 7 and 9; right arrow) which diminishes markedly in intensity in cells treated with a monoclonal antibody reactive with Fas (lanes 2, 4, 6, 8, 10).

Many autoantigens are cleaved by caspases during apoptosis [5], and we hypothesized that this 58-kDa protein might also be cleaved during Fas-mediated cell death. Review of the literature identified the autoantigen vimentin as a candidate 58-kDa protein which has been reported to be targeted by caspases during programmed cell death [34]. To determine if the 58-kDa protein immunoprecipitated by the monoclonal antibodies was vimentin, we solubilized Jurkat cells in Nonidet P-40 lysis buffer and immunoprecipitated using the monoclonal antibodies Z2, Z4, Z6, Z12 and Z14 (Figure 8). The immunoprecipitates were subjected to 7% SDS–PAGE, transferred to nitrocellulose and immunoblotted using monoclonal
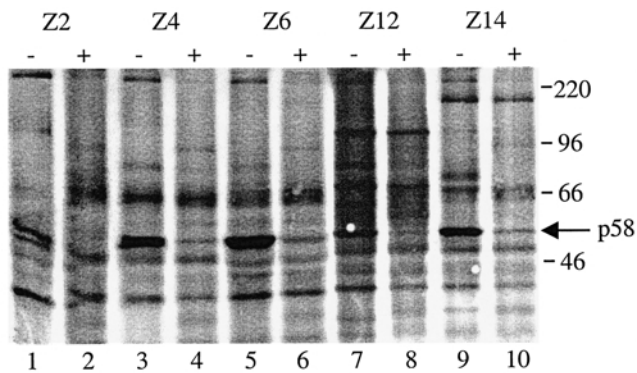
Figure 7. Selected monoclonal antibodies precipitate a 58 kDa protein (p58) from ^{35}S labelled non-apoptotic Jurkat lysates which is diminished in labelled apoptotic immunoprecipitates. Jurkat cells were labelled with ^{35}S methionine and cysteine and lysed either before (−) or 3 h after (+) the addition of anti-Fas 7C11. Proteins were then precipitated using selected monoclonal antibodies, separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. A band corresponding to the 58 kDa protein (p58) is indicated at the right side of the panel. The relative migration of molecular mass markers in kilodaltons is indicated on the right side of the panel. Lanes are numbered at the bottom of the panel. Antibodies correspond to those listed in Table 1.
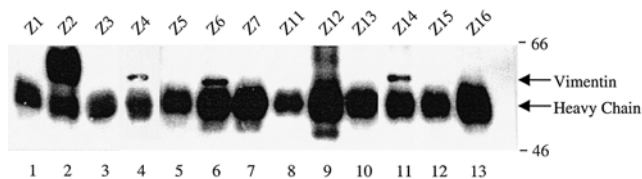

Figure 8. Monoclonal antibodies immunoprecipitate vimentin from Jurkat cell lysates. Jurkat cells were solubilized in Nonidet P-40 lysis buffer and immunoprecipitated with individual monoclonal antibodies. After separation on a 7% SDS-polyacrylamide gel and transfer to nitrocellulose, the filter was immunoblotted with anti-vimentin antibody. A band corresponding to vimentin is indicated at the right side of the panel. The relative migration of molecular mass markers in kilodaltons is indicated on the right side of the panel. Lanes are numbered at the bottom of the panel. Antibodies correspond to those listed in Table 1.

anti-vimentin antibody (Sigma Chemical Co.). As shown in Figure 8, a 58-kDa protein is recognized by anti-vimentin antibodies in immunoprecipitates using Z4, Z6, Z12 and Z14 (lanes 5, 6, 9 and 11). Monoclonal antibody Z2 (lane 2) precipitates a protein at a position slightly higher than that predicted for vimentin. The high signal intensity extends into and obscures the area in which vimentin is expected to migrate. These results correspond exactly with those monoclonal antibodies which immunoprecipitate the 58-kDa protein from the ^{35}S metabolic labelling experiments (Figures 2 and 7). When taken together, these results suggest that the autoantigen vimentin is the target of a subset of monoclonal antibodies generated by immunizing mice with apoptotic Jurkat cells.

Discussion

Several autoimmune diseases including systemic lupus erythematosus (SLE), Sjögren's syndrome, polymyositis, and scleroderma are characterized by the production of autoantibodies [reviewed in 35]. Although the initiating events in autoantibody production remain unknown, there is increasing evidence that apoptotic cells may serve as reservoirs of autoantigens. Human keratinocytes induced to undergo apoptosis demonstrate clustering of autoantigens into discreet blebs at the cell surface [3]. Moreover, cultured keratinocytes exposed to ultraviolet B/ ultraviolet A irradiation (a known apoptotic stimulus) demonstrate enhanced binding of autoantibodies directed against Sm, RNP, Ro, and La to the cell surface membrane [2]. Such evidence raises the possibility that apoptotic cells might present self-antigens to the immune system in a manner that bypasses normal mechanisms of tolerance.

More recently, there has been further support for a role for apoptosis in the development of autoantibodies. Normal mice immunized intravenously with syngeneic apoptotic thymocytes develop low levels of autoantibodies whereas similar mice immunized with non-apoptotic syngeneic splenocytes generally do not develop autoantibodies [21]. Our study differs from Mevorach *et al.* in several potentially important ways. First, the route of immunization differed in that we immunized intraperitoneally or subcutaneously rather than intravenously. The route of immunization could have a considerable effect on antigen processing and the subsequent immune response, which at present is not well understood. Second, our study utilized monoclonal antibodies rather than sera derived from mice immunized using apoptotic cells, and included analysis of immunoprecipitates of cell lysates using these monoclonal antibodies. These methodological differences may account for the ability of our monoclonal antibodies to recognize ribosomes and snRNPs, while the study of Mevorach *et al.* failed to identify such antibodies.

Perhaps the most important difference between our study and that of Mevorach *et al.* is that we used human rather than syngeneic cells to immunize our mice. We acknowledge that the possibility exists that the antibodies produced in our mice are simply the result of xenogeneic differences between mice and humans. However, there are several observations that suggest this is not the case. First, previous immunization studies in our laboratory using intracellular human antigens (i.e., produced by permeabilizing Jurkat cells with digitonin, followed by immunization and production of monoclonal antibodies) identified a number of intracellular antigens, including TIA-1 and the T cell receptor ξ chain [24, 25]. Importantly, none of these monoclonal antibodies recognized prominent autoantigens, as was observed in our present study. Second, the fact that nearly half of the monoclonal antibodies we screened precipitate known autoantigens suggests an intriguing preference by the immune response for these normally inaccessible intracellular proteins. Such a bias might be related to enhanced

access to these autoantigens in the surface blebs of apoptotic cells or through the creation of neoepitopes through post-translational modifications occurring during programmed cell death (see below) [3, 36–38]. Regardless of the methodological differences that exist between our study and that of Mevorach *et al.*, the monoclonal antibodies report herein will be extremely useful probes to study human autoantigens, particularly Ku, which has been difficult to study because of the lack of antibodies capable of recognizing the intact Ku/DNA-PK complex.

Our results confirm that proteins translocated to the cell surface of apoptotic cells may be preferentially targeted by the immune system. Two distinct populations of surface blebs are present in apoptotic cells [39]. 'Small blebs' contain ribosomal antigens, Ro, signal recognition particle (SRP) and calreticulin while 'apoptotic bodies' contain multiple autoantigens including snRNPs, Sm, Ku/DNA-PK, PARP, and nuclear mitotic apparatus protein (NuMA) [40]. Nearly half of the monoclonal antibodies we screened from normal mice immunized with apoptotic Jurkat cells precipitate autoantigens present in these apoptotic blebs, including Ku, ribosomes and snRNPs. To our knowledge, apoptotic cells have not been examined for the presence of vimentin within surface blebs.

It has been postulated that posttranslational modifications of proteins during apoptosis may lead to the presentation of modified proteins in such a way that normal mechanisms of tolerance are bypassed [reviewed in 4, 5]. Many autoantigens are substrates for cleavage by caspases during apoptosis, including DNA-PK, U1-70ukDa, and vimentin [34, 36, 38]. Other types of posttranslational modifications might also predispose to autoantibody formation including ubiquitination, transglutamination, phosphorylation, and dephosphorylation [reviewed in 4, 5]. Finally, cleavage of several autoantigens by granzyme B has been observed to occur at unique sites not recognized by caspases [8, 9]. Interestingly, DNA-PK, Ku-70, and U1-70 kDa were found to be substrates for granzyme B in the study of Casciola-Rosen *et al.*, raising the intruiging possibility that granzyme-mediated autoantigen cleavage may be involved in bypassing tolerance to the Ku/DNA-PK and U1-snRNP complexes by creating novel epitopes that are not generated during other forms of cell death.

Since all four of the proteins or protein complexes recognized by the monoclonal antibodies in our present study are known to be modified in some way during apoptosis, these results provide further indirect evidence for a relationship between autoantigen modifications occurring during apoptosis and the development of autoimmunity. First, two monoclonal antibodies directly recognize Ku-70. DNA-PK, a constituent of the Ku complex, is cleaved by caspases [36], and both Ku-70 and DNA-PK are substrates for granzyme B [8]. The proteolysis of DNA-PK could result in neoepitope formation followed by the subsequent presentation to T cells not tolerant to the novel peptide, thus resulting in an immune response. By the process of 'intermolecular epitope spreading' in which an immune response to a particular protein within a complex facilitates antibody formation against other proteins in the same complex, it is conceivable that Ku-70 could also become a target, as has been described for the immune response against the U1-snRNP complex and the Ro/La particle [41].

Two of the monoclonal antibodies produced by immunizing mice with apoptotic Jurkat cells precipitate ribosomes, including the ribosomal P autoantigens. The ribosomal P proteins have not been reported to undergo posttranslational modifications during apoptosis, but 28S rRNA is cleaved at specific sites during programmed cell death [11, 42]. Interestingly, three-quarters of patients with SLE whose sera contains antiribosomal P antibodies also have antibodies to a region of 28S rRNA [43]. This association between antiribosomal P antibodies and anti-28S rRNA antibodies is in accord with the hypothesis that an immune response against neoepitopes formed following cleavage of 28S rRNA results in 'epitope spreading' to other ribosomal components (e.g. ribosomal P proteins).

Four of the monoclonal antibodies derived from mice immunized with apoptotic cells precipitated snRNPs. As previously mentioned, the U1-snRNP undergoes a number of changes during apoptosis including association with phosphorylated SR proteins [17, 18], caspase-mediated cleavage of the U1-70 kDa protein [38], and cleavage of the U1-snRNA molecule [10]. Each of these posttranslational modifications could result in neoepitope formation and a subsequent immune response. Finally, five of the monoclonal antibodies precipitate the intermediate filament vimentin, a known caspase substrate. Autoantibodies to intermediate filaments have been described in several autoimmune diseases including SLE, Behçet's disease, rheumatoid arthritis, and Sjögren's syndrome [44–46]. In a study of patients with SLE, over half were found to have antibodies specifically targeting vimentin [45].

In summary, by exposing mice to apoptotic human lymphocytes we have developed a panel of monoclonal antibodies, many of which specifically recognize at least four distinct autoantigens or particles. These monoclonal antibodies may be useful tools for identifying other components of autoantigen particles such as the Ku/DNA-PK complex and U-snRNPs. Importantly, these experiments solidify the intriguing link between apoptosis, posttranslational protein modifications, and the subsequent development of an autoimmune response [47–49]. Future experiments in which monoclonal antibodies are derived from mice immunized with apoptotic syngeneic cells or cells exposed to Granzyme B are underway, with the long term goal to better understand the mechanism(s) by which tolerance to human autoantigens is broken.

Acknowledgements

References

1. LeFeber W.P., Norris D.A., Ryan S.R., Huff J.C., Lee L.A., Kubo M., Boyce S.T., Kotzin B.L., Weston W.L. 1984. Ultraviolet light induces binding of antibodies to selected nuclear antigens on cultured keratinocytes. *J. Clin. Invest.* **74:** 1545–1551

2. Golan T.D., Elkon K.B., Gharavi A.E., Krueger J.G. 1992. Enhanced membrane binding of autoantibodies to cultured keratinocytes of systemic lupus erythematosus patients after ultraviolet B/ultraviolet A irradiation. *J. Clin. Invest.* **90:** 1067–1076

3. Casciola-Rosen L.A., Anhalt G., Rosen A. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface blebs on cultured keratinocytes. *J. Exp. Med.* **179:** 1317–1330

4. Rosen A., Casciola-Rosen L.A. 1999. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. *Cell Death Diff.* **6:** 6–12

5. Utz P.J., Anderson P. 1998. Posttranslational modifications, apoptosis, and bypass of tolerance to autoantigens. *Arthritis Rheum.* **41:** 1152–1160

6. Nicholson D., Thornberry N. 1997. Caspases: killer proteases. *TIBS* **22:** 299–306

7. Alnemri E., Livingston D., Nicholson D., Salveson G., Thornberry N., Wong W., Yuan J. 1996. Human ICE/CED-3 protease nomenclature. *Cell* **87:** 171

8. Andrade F., Roy S., Nicholson D., Thornberry N., Rosen A., Casciola-Rosen L.A. 1998. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity* **8:** 451–460

9. Casciola-Rosen L.A., Andrade F., Ulanet D., Wong W., Rosen A. 1999. Cleavage by granzyme B is strongly predictive of autoantigen status. Implications for initiation of autoimmunity. *J. Exp. Med.* **190:** 815–826

10. Degen W.G.J., van Aarssen Y., Piepers P., Pruijn G.J.M., Utz P.J., van Venrooij W.J. 2000. The fate of the U1 small nuclear RNP complex during anti-Fas induced apoptosis: specific cleavage of the U1 snRNA molecule. *Cell Death Differ.* **7:** 70–79

11. Houge G., Robaye B., Eikhom T., Golstein J., Mellgren G., Gjertsen B., Lanotte M., Doskeland S. 1995. Fine mapping of 28S rRNA sites specifically cleaved in cells undergoing apoptosis. *Mol. Cell Biol.* **15:** 2051–2062

12. Rutjes S.A., van der Heijden A., Utz P.J., van Venrooij W.J., Pruijn G.J.M. 1999. Nucleolytic degradation of the

13. Rumore P., Steinman C. 1990. Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone. *J. Clin. Invest.* **86:** 69–74

14. Huck S., Deveaud E., Namane A., Zouali M. 1999. Abnormal DNA methylation and deoxycytosine-deoxyguanine content in nucleosomes from lymphocytes undergoing apoptosis. *FASEB J.* **13:** 1415–1422

15. Autuori F., Farrace M., Oliverio S., Piredda L., Piacentini M. 1998. 'Tissue' transglutaminase and apoptosis. *Adv. Bioch. Engineer* **62:** 130–136

16. Asaga H., Yamada M., Senshu T. 1998. Selective deimination of vimentin in calcium ionophore-induced apoptosis of mouse peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **243:** 641–646

17. Utz P.J., Hottelet M., Schur P., Anderson P. 1997. Proteins phosphorylated during stress-induced apoptosis are common targets for autoantibody production in patients with systemic lupus erythematosus. *J. Exp. Med.* **185:** 843–854

18. Utz P.J., Hottelet M., van Venrooij W.J., Anderson P. 1998. Association of phosphorylated SR proteins and the U1-small nuclear ribonuclear protein autoantigen complex accompanies apoptotic cell death. *J. Exp. Med.* **187:** 547–560

19. Rutjes S.A., Utz P.J., Broekhuis C., van der Heijden A., van Venrooij W.J., Pruijn G.J.M. 1999. The La (SS-B) autoantigen, a key protein in RNA biogenesis, is dephosphorylated and cleaved early during apoptosis. *Cell Death Differ.* **6:** 976–986

20. Marushige Y., Marushige K. 1995. Disappearance of ubiquitinated histone H2A during chromatin condensation in TGFβ1-induced apoptosis. *Anticancer Res.* **15:** 267–272

21. Mevorach D., Zhou J., Song X., Elkon K. 1998. Systemic exposure to irradiated apoptotic cells induces autoantibody production. *J. Exp. Med.* **188:** 387–392

22. Zhang C., Ao A., Seth A., Schlossman S. 1996. A mitochondrial membrane protein defined by a novel monoclonal antibody is preferentially detected in apoptotic Jurkat cells. *J. Immunol.* **157:** 3980–3987

23. Kohler G., Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:** 495–497

24. Anderson P., Blue M., O'Brien C., Schlossman S. 1989. Monoclonal antibodies reactive with the T cell receptor ξ chain: production and characterization using a new method. *J. Immunol.* **143:** 1899–1904

25. Anderson P., Nagler-Anderson C., O'Brien C., Levine H., Watkins S., Slayter H., Blue M., Schlossman S. 1990. A monoclonal antibody reactive with a 15-kDa cytoplasmic granule-associated protein defines a subpopulation of CD8+ T lymphocytes. *J. Immunol.* **144:** 574–582

26. Karwan R., Bennett J.L., Clayton D.A. 1991. Nuclear RNase MRP processes RNA at multiple discrete sites: interaction with an upstream G box is required for subsequent downstream cleavages. *Genes Dev.* **5:** 1264–1276

27. Habets W., Hoet M., de Jong B., van der Kamp A., van Venrooij W.J. 1989. Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with

small cytoplasmic Y RNAs during apoptosis. *J. Biol. Chem.* **274:** 24799–24807

human autoantibodies as well as with experimentally-induced mouse monoclonal antibodies. *J. Immunol.* **143**: 2560–2566

28. Laemmli E. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685

29. Pearson W., Lipman D. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci., USA* **85**: 2444–2448

30. Francoeur A., Peebles C., Heckman K., Lee J., Tan E.M. 1985. Identification of ribosomal autoantigens. *J. Immunol.* **135**: 2378–2384

31. Overzet K., Gensler T., Kim S., Geiger M., van Venrooij W.J., Pollard K.M., Anderson P., Utz P.J. 1999. Small nucleolar ribonucleoprotein autoantigens associate with phosphorylated serine arginine (SR) splicing factors during apoptosis. *Arthritis Rheum.* **43**: 1327–1335

32. Smolen J., Butcher B., Fritzler M., Gordon T., Hardin J., Kalden J., Lahita R., Maini R., Reeves W., Reichlin M., Rothfield N., Takasaki Y., van Venrooij W.J., Tan E.M. 1997. Reference sera for antinuclear antibodies. *Arthritis Rheum.* **40**: 413–418

33. Satoh M., Reeves W. 1994. Induction of lupus-associated autoantibodies in BALB/c mice by intraperitoneal injection of pristane. *J. Exp. Med.* **180**: 2341–2346

34. Prasad S., Thraves P., Kuettel M., Srinivasarao G., Dritschilo A., Soldatenkov V. 1998. Apoptosis-associated proteolysis of vimentin in human prostate epithelial tumor cells. *Bioch. Biophys. Res. Commun.* **249**: 332–338

35. von Mühlen C.A., Tan E.M. 1995. Autoantibodies in the diagnosis of systemic rheumatic diseases. *Semin. Arthritis Rheum.* **24**: 323–358

36. Casciola-Rosen L.A., Anhalt G.J., Rosen A. 1995. DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J. Exp. Med.* **182**: 1625–1634

37. Casciola-Rosen L.A., Nicholson D.W., Chong T., Rowan K.R., Thornberry N.A., Miller D.K., Rosen A. 1996. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J. Exp. Med.* **183**: 1957–1964

38. Casciola-Rosen L.A., Miller D.K., Anhalt G.J., Rosen A. 1994. Specific cleavave of the 70 kDa protein component of the U1 small nuclear riboprotein is a characteristic biochemical feature of apoptotic cell death. *J. Biol. Chem.* **269**: 30757–30760

39. Casciola-Rosen L.A., Rosen A., Petri M., Schlissel M. 1996. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. *Proc. Natl. Acad. Sci., USA* **93**: 1624–1629

40. Casciola-Rosen L.A., Rosen A. 1997. Ultraviolet light-induced keratinocyte apoptosis: a potential mechanism for the induction of skin lesions and autoantibody production in LE. *Lupus* **6**: 175–180

41. Craft J., Fatenejad S. 1997. Self antigens and epitope spreading in systemic autoimmunity. *Arthritis Rheum.* **40**: 1374–1382

42. Delic J., Copper-Moisan M., Magdelenat H. 1993. Gamma ray-induced transcription and apoptosis-associated loss of 28S rRNA in interphase lymphocytes. *Int. J. Rad. Biol.* **64**: 39–46

43. Chu J., Brot N., Weissbach H., Elkon K. 1991. Lupus antiribosomal P antisera contain antibodies to a small fragment of 28S rRNA located in the proposed ribosomal GTPase center. *J. Exp. Med.* **174**: 507–514

44. Akoglu T., Kozakoglu H., Akoglu E. 1986. Antibody to intermediate filaments of the cytoskeleton in patients with Behçet's disease. *Clin. Immunol. and Immunopath.* **41**: 427–432

45. Alcover A., Molano J., Renart J., Gil-Aguado A., Nieto A., Avila J. 1984. Antibodies to vimentin intermediate filaments in sera from patients with systemic lupus erythematosus. *Arthritis Rheum.* **27**: 922–928

46. Kurki P., Helve T., Virtanen I. 1983. Antibodies to cytoplasmic intermediate filaments in rheumatic diseases. *J. Rheum.* **10**: 558–562

47. Utz P.J., Gensler T.J., Anderson P. 2000. Death, autoantigen modifications, and tolerance. *Arthritis Research* **2**: 101–114

48. Neugebauer K.M., Merrill J.T., Wener M.H., Lahita R.G., Roth M.B. 2000. SR proteins are autoantigens in patients with systemic lupus erythematosus: importance of phosphoepitopes. *Arthritis Rheum.* **43**: 1768–1778

49. Greidinger E.L., Casciola-Rosen L., Morris S.M., Hoffman R.W., Rosen A. 2000. Autoantibody recognition of distinctly modified forms of the U1-70-kd antigen is associated with different clinical disease manifestations. *Arthritis Rheum.* **43**: 881–888